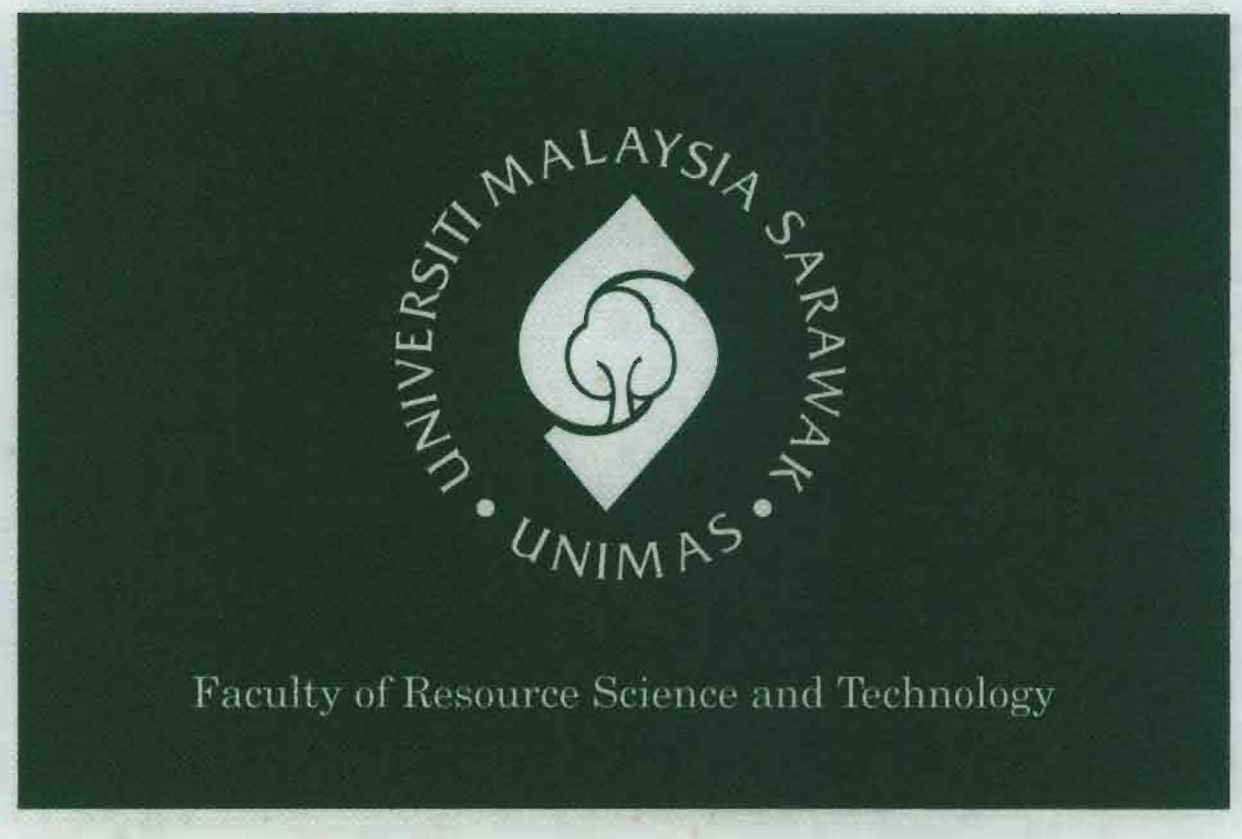

POPULATION GENETIC STRUCTURE OF *Hampala macrolepidota* (Cyprinidae) IN SEVERAL POPULATIONS IN MALAYSIA USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

Die Ling Ling

Bachelor of Science with Honours
(Animal Resource Science and Management)
2005

POPULATION GENETIC STRUCTURE OF *Hampala macrolepidota* (Cyprinidae) IN SEVERAL POPULATIONS IN MALAYSIA USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

DIE LING LING

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours
(Animal Resource Science and Management Program)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2005

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

Die Ling Ling
Program of Animal Resource Science and Management
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

AKNOWLEGMENT

I especially want to thank Mr. Yuzine who inspired me to undertake the study. Besides that, I also thank to all the lecturers involved, for their thoughtful and advise during the completion of the project. This research was part of the Unimas Student research grant entitled "Population Genetic Structure of *Hampala maclolepidota* (Cyprinidae) in several populations in Malaysia using RAPD Technique" (Project no 245/2001 (4)).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Population genetic structure of *Hampala macrolepidota* (Cyprinidae) in several populations in Malaysia using Random Amplified Polymorphic DNA (RAPD) Technique

Die Ling Ling

Animal Resource Science and Management
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

This study examined thirty individuals of *Hampala macrolepidota* (Bleeker) using Random amplified polymorphic DNA (RAPD) – PCR technique. Samples were collected from ten different locations in Malaysia. The RAPD study identified five primers useful for population analysis. 37.50% of the RAPD loci were polymorhic. Mean Nei's (1973) gene diversity was 0.1016. While, mean G_{ST} value across loci was 0.735, and the average for *Nm* values was 0.1798. The population's genetic distance was similar with the observed individual's pair-wise distance. The close relationship between *H. macrolepidota* samples was indicated by their low pair-wise genetic distance. In addition, the high percentage of sharing of banding profiles obtained also suggested that the *H. macrolepidota* samples from Peninsular Malaysia were closely related to the samples from Sarawak.

Keywords: Population genetic structure, *Hampala macrolepidota*, RAPD

ABSTRAK

Dalam kajian ini, tiga puluh individu bagi Hampala macrolepidota (Bleeker) telah dikaji dengan menggunakan teknik analisis secara rawak DNA polimorfik (RAPD) -PCR. Semua sampel dikumpul dari sepuluh lokasi yang berlainan di Malaysia. Kajian RAPD ini telah mengenali lima primer yang berguna untuk menganalisiskan populasi. 37.50% bagi RAPD "loci" adalah polimorfik. Purata bagi Nei's (1973) kebagaian gen adalah 0.1016. Manakala, purata bagi nilai G_{ST} yang melalui "loci" adalah 0.735, serta memberi nilai purata yang dianggarkan bagi Nm iaitu 0.1798. Jarak genetik bagi populasi adalah hampir sama dengan jarak 'pair-wise' bagi individu yang diperhatikan. Perhubungan yang rapat di antara sampel H. macrolepidota adalah ditanda oleh jarak genetik mereka yang rendah. Tambahan pula, profil bagi belang yang mempunyai peratusan yang tinggi didapati juga mencadangkan bahawa sampel bagi H. macrolepidota dari Semenajung Malaysia mempunyai perhubungan yang rapat dengan sampel dari Sarawak.

Kata kunci: Struktur populasi, Hampala macrolepidota , RAPD

1.0 INTRODUCTION

The freshwater fishes of the genus *Hampala* (Bleeker) belong to the family Cyprinidae. There are currently three described species of *Hampala* in Malaysia: *Hampala macrolepidota*, *Hampala bimaculata*, and *Hampala sabana* (Inger and Chin 1962; Mohsin and Ambak 1991; Roberts 1979).

H. macrolepidota is locally known as Sebarau fish in Peninsular Malaysia and Adong fish in Sarawak. The vertical black band on side below readily identifies it dorsal. The anterior rays of dorsal are usually dusky. Mostly, the black markings disappear on the sides when the fish is about 300mm long (Inger & Chin 1962). The habitat for this fish is in clear water with sands and rocks at the bottom compared to muddy areas. The juveniles only feed on some insects and others. Meanwhile, the adult are carnivorous and feed on other fishes (Inger & Chin 1962). *H. macrolepidota* is considered as one of the important sport fish and highly priced in Malaysia.

H. macrolepidota is the most widespread *Hampala* form in Malaysia and it was the only *Hampala* species currently described from Peninsular Malaysia (Mohsin & Ambak 1983). This primary freshwater fish species may be effected by glacial during the Pleistocene period. Thus, the wide geographically distribution of *H. macrolepidota* make it suitable candidate for studying genetic variation in freshwater fish in relation to the biogeographically history of Sundaland.

During the Pleistocene (glacial maxima), the sea level was lowered; Peninsular Malaysia, Borneo and Sumatra were drained by a big river system called the North Sunda River. The North Sunda

River was rich with freshwater fish fauna dominated by the cyprinids (Mohsin & Ambak 1983). So, freshwater fish of Peninsular Malaysia and Borneo Island may have intermixed in these lowlands during this period. During the late Pleistocene (10,000- 20,000 years ago), the rising of sea level caused the great river system to submerge 40 to 100 meters under the surface of water while Borneo Island was separated from mainland Asia (Mohsin & Ambak 1983). So, it is important to test the population genetic of *H. macrolepidota* that may have been affected by glaciations during the Pleistocene low sea levels.

Genetic drift is one of the major sources of genetic variation between populations (Halliday 1993). Therefore, we predicted that genetic drift may play an important role in the genetic variation between *H. macrolepidota* from Peninsular Malaysia with populations from the Borneo Island (Sarawak). Previous study by Jefferine and Esa (2003) on phylogenetic analysis of *Hamapala* in Sarawak using *cytochrome b* mitochondrial DNA (mtDNA) found high mtDNA similarities between samples of *H. macrolepidota* from the southern region of Sarawak and *H. macrolepidota* from Peninsular Malaysia, supporting the recent isolated of the Borneo from mainland Asia (Peninsular Malaysia) during the last Pleistocene (10,000-20,000 years ago).

RAPD is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al.* 1990; Weish and McClelland 1990). RAPD produces DNA profiles of varying complexity, depending on the primer and template used. Each amplification product is expected to result from the existence of two annealing sites in inverted orientations, 3' ends facing each other, within amplifiable distance.

Among all the technique, RAPD is one of the useful technique to assess genetic variation in fish population and used in fishery management and conservation genetic of wild populations (Williams *et al.* 1990).

However, RAPD analysis has some limitations which must be carefully considered. It follows a dominant marker/pattern where a homozygote allele cannot be distinguished from a heterozygote allele. In addition, it is unable to assign bands to specific loci unless a previous pedigree analysis is performed. In applying this method, it is assumed that populations are under the Hardy-Weinberg equilibrium, which polymorphic bands segregate in the Mendelian way, and that marker alleles from different loci do not co-migrate to the same position in the gel (D'Amato *et al.* 1996). Basically, 10-mer primers with 50- 80% G+C content are more preferred to use (Guang *et al.* 1996).

Therefore, study was done to provide insight into the population structure and biogeography of *H. macrolepidota* from a nuclear gene viewpoint. The RAPD technique was selected to use in this study because it has been shown to have a high power of resolution, especially in detecting cryptic pairs of species and in confirming close relationships between species (Lehmann *et al.* 2000; Bartish *et al.* 2000). Other studies using RAPD analysis such as on the *Prochilodus marggravii* (Terumi *et al.* 2002) and *Silurus asotus* (Jong and Gye 2001) have shown the usefulness and effectiveness of this molecular analysis in the genetic diversity of freshwater fish studies.

2.0 OBJECTIVES

The aims of the study are to examine the level of genetic variation in several population of *Hampala macrolepidota* by using RAPD technique and also to compare the results with those done using *cytochrome b* mtDNA by Jefferine and Esa (2003).

.

3.0 MATERIALS METHODS

3.1 Sample Collection and Preservation

A total of 30 individuals of *H. macrolepidota* (see Figure 1) were collected from 10 populations in Peninsular Malaysia and Sarawak (see Figure 2; Table 1). The samples were preserved in 95% ethanol and stored at -20°C fridge for long term - storage until used for genetic study.

Family : Cyprinidae
Order : Cypriniformes
Genus : *Hampala*
Species : *Hampala macrolepidota*

Figure 1. *Hampala macrolepidota*

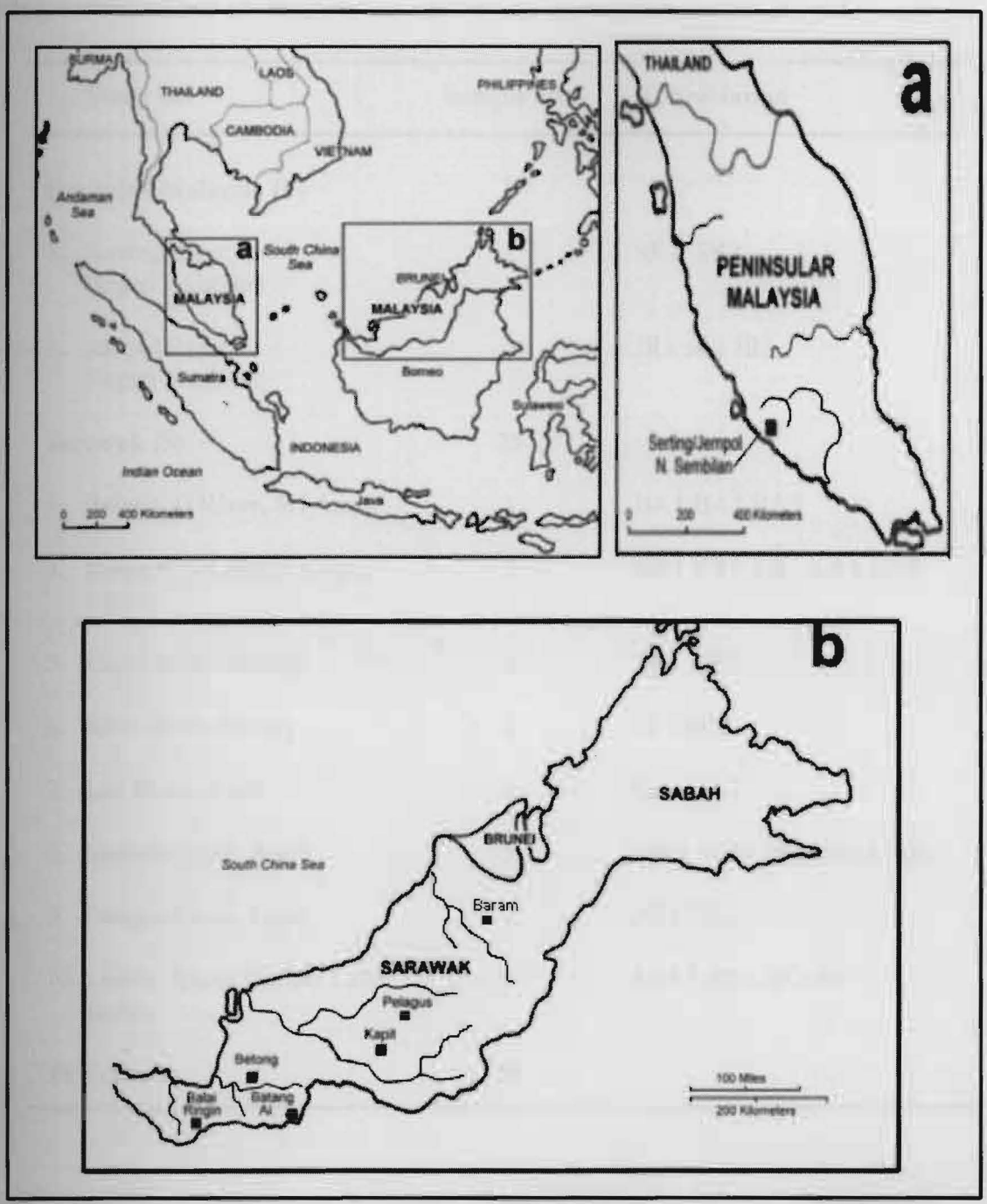

Figure 2. Location map of *H. macrolepidota* samples used in this study.

Table 1. Samples locations and sample sizes of *Hampala macrolepidota* used in the study

Study site	Sample size	Abbreviation
Penisular Malaysia (P)	**5**	
1. Serting River, Negeri Sembilan	2	SR1 SR2
2. Jempol River, Negeri Sembilan	3	JR1 JR2 JR3
Sarawak (S)	**25**	
3. Batang Ai River, Sri Aman	3	BA1 BA2 BA3
4. Krang River, Balai Ringin Serian	5	KR1 KR2 KR3 KR4 KR5
5. Layar River, Betong	2	LR1 LR2
6. Spak River, Betong	2	SP1 SP2
7. Lan River, Kapit	2	LA1 LA2
8. Menuan River, Kapit	5	MR1 MR2 MR3 MR4 MR5
9. Pelagus River, Kapit	2	PR1 PR2
10. Loagan Bunut Oxbow Lake, Baram	4	LB1 LB2 LB3 LB4
TOTAL	**30**	

3.2 DNA extraction and Electrophoresis

Genomic DNA from muscle tissue was extracted using a modified Cetyl tri-methylammonium bromide (CTAB) protocol (Grewe *et al.* 1993) with the presence of Proteinase-K. Pelleted DNA was re- dissolved in 100µL of sterilized distilled water. The quality and approximate yield was analyzed by electrophoresis in 1% agarose gel, which contains ethidium bromide at 90 V for 30 min.

3.3 Primers

Thirty arbitrary decamer primers (Operon Technologies, Alameda, California, USA), with a GC – content between 60 and 70%, were used determine genetic differences among the populations of *H. macrolepidota* (see Table 2), according to the descriptions of William *et al.*, (1990). PCR amplifications were carried out in a total reaction volume of 24µl.

Table 2. Primers (Operon Technologies, Alameda, California, USA) and primers sequences used in this study.

Primer	Primer Sequence (5' to 3')	GC- content [%]
OP A – 01	CAGGCCCTTC	70
OPA – 02	TGCCGAGCTG	70
OPA – 03	AGTCAGCCAC	60
OPA – 04	AATCGGGCTG	60
OPA – 05	AGGGGTCTTG	60
OPA – 06	GGTCCCTGAC	70
OPA – 07	GAAACGGGTG	60
OPA – 08	GGTCCCTGAG	70
OPA – 09	GGGTAACGCC	70
OPA – 10	GTGATCGCAG	60

Table 2. Continued

Primer	Primer Sequence (5' to 3')	GC- content [%]
OP A –13	CAGCACCCAC	70
OPA – 15	TTCCGAACCC	60
OPA – 17	GACCGCTTGT	60
OPA – 18	AGGTGACCGT	60
OPA – 20	GTTGCGATCC	60
OPD – 01	GGTCCCTGAC	70
OPD – 02	GGACCCAACC	70
OPD – 04	TCTGGTGAGG	60
OPD – 06	ACCTGAACGG	60
OPD – 07	TTGGCACGGG	70
OPD – 09	CTCTGGAGAC	60
OPD – 10	GGTCTACACC	60
OPD – 11	AGCGCCATTG	60
OPD – 12	CACCGTATCC	60
OPD – 13	GGGGTGACGA	70
OPD – 14	CTTCCCCAAG	60
OPD – 15	CATCCGTGCT	60
OPD – 16	AGGGCGTAAG	60
OPD – 17	TTTCCCACGG	60
OPD – 18	GAGAGCCAAC	60

3.4 Random Amplified Polymorphic DNA (RAPD) - Polymerase Chain Reaction (PCR)

RAPD reactions were carried out based on the methods developed by William *et al.* (1990). The content of the reaction mixtures for RAPD – PCR and their appropriate amount were described in Table 3.

Table 3. RAPD – PCR mixtures contents

Component	1x reaction	10 x reactions
H_2O (RO, sterile)	17.3	173.0
10x Reaction buffer	2.5	25.0
$MgCl_2$	1.5	15.0
dNTP mix (10 mM)	0.5	5.0
Primer (10M)	1.0	10.0
Template DNA (10ng/L)	1.0	****
Taq polymerase (5units/L)	0.2	2.0

A negative control (without template DNA) was included in each set of reactions amplified. All reactions were carried out on a programmable thermal cycler (Biometra T – personal). The cycle parameters were described in Table 4.

Table 4. The cycle parameters

Step	Temp	Time	No cycles
Denaturation	94°C	2 min	
Annealing	34°C	30s	
Extension	72°C	2min	1
Denaturation	94°C	15s	
Annealing	34°C	30s	40
Extension	72°C	2min	
Extension	72°C	5min	
Soak	4°C	5min	1

Aliqouts (3µl) of the reaction products were separated by electrophoresis using a 1.5% agarose gel containing ethidium bromide. Fragement sizes were estimated by comparison to a molecular weight marker DNA (Mass Ruler ^{TM} DNA Ladder Low Range). In each case, electrophoresis was run for approximately 30 minutes at 80V and photographed under UV light.

3.5 Data Analysis

RAPD banding profile obtained were analyzed with the RAPDistance Program Package Version 1.04 (Australia National University, Canberra). RAPD products were scored as presence (1) or absence (0) of each applicant evaluated. All clear fragments of the same length were recorded as one genetic character. The Pair-wise distance for individuals was done base on the Dice formulation (Nei and Li 1979) and performed a hierarhical dendrogram to determine the genetic similarities within individuals of *H .macrolepidota* using the neighbor-joining algorithm.

Additionally, for comparative purposes, a data matrix of individuals' marker containing the band scoring information was transformed to allele frequencies under the assumption that each amplified band corresponds to a different RAPD locus (Appendix 1). The dataset also used to calculate genetic distance (Nei's unbiased distance estimate, Nei 1978) among population and construct a dendrogram using POPGENE (Population Genetic Analysis) Version 1.31 software package of Yeh and Boyle (1997). For each RAPD locus, allele frequencies across all populations were tested using X^2 in POPGENE to estimate the populations' homogeneity. Furthermore, G_{ST} for RAPD loci was calculated to evaluate the degree of genetic subdivision among populations. From these values, the estimation of gene flow ($Nm= 0.5(1- G_{ST})/ G_{ST}$) for each locus was derived. The non parametric Kruskal-Wallis test (Lehmann 1975) was carried out to test the significance of similarity coefficient for each locus between the sampling sites.

4.0 RESULTS

4.1 Analysis of PCR amplification profiles

For initial screening, thirty random primers (Operon, sets A and D; see Table 2) were tested to evaluate the amplification product. However, only ten primers produced amplification products and only five primers (OPA-01, OPA-08, OPA-10, OPA-18 and OPA-20) from Operon Technologies Primer Kit A generated unambiguously RAPD bands. In this study, the similarity of RAPD bands analyzed was base on the number of shared amplification products using a simple matching method. A total of 430 bands were scored in the total RAPD profiles. Product sizes ranged from 300 up to 900 base pair (bp). Repeatability of the amplification reactions were conducted at least two time to ensure the consistency of the bands pattern. Figure 3 and 4 shows the example of the band pattern generated with the primer OPA-20.

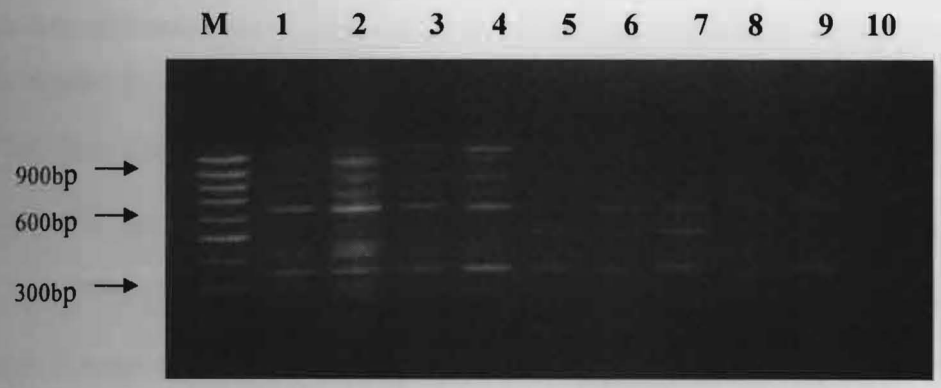

Figure 3. Specific RAPD patterns of *H. macrolepidota* amplified by arbitrary primer OPA-20 (GTTGCGATCC). Lane M represents size marker (Mass Ruler ™ DNA Ladder Low Range). Lane 1 and 2 show individual DNA samples from Spak River (SP1 and SP2). Lane 3 and 4 represent individual DNA samples from Lan River (LA1 and LA2). Lane 5, 6 and 7 represent individual DNA samples from Jempol River (JR1, JR2 and JR3). Lane 8 and 9 show individual DNA samples from Layar River (LR1 and LR2). Lane 10 represents negative control.

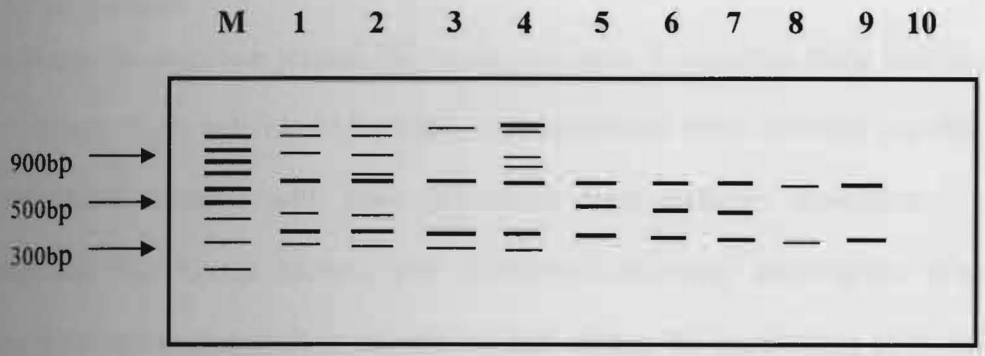

Figure 4. Illustration of the genetic profiles of *H. macrolepidota* using primer OPA-20 (GTTGCGATCC).

4.2 Pair-wise Distance

Genetic similarities between individuals and populations of *H. macrolepidota* were high based on the pair-wise distance. Table 5 showed the genetic distance among individuals. A minimum distance (0.00) was found between samples pairs from Layar River (LR1) and Serting River (SR1); Spak River (SP2) and SR1; Loagan Bunut (LB1) and SR1; LB1and LR1; LB1 and SP2, a maximum distance (0.257) was found between the Spak River (SP1) and Krang River (KR2). Meanwhile, Table 6 summarizes the genetic distance statistics (Nei's unbiased estimate; Nei 1978) among the populations studied. The genetic distance estimates range in the value of 0.00 (between SR and MR, SR and PR, MR and PR) to 0.2257 (between LR and LA). The estimation range values for population's genetic distance are similar to the observed individual's pair-wise distance.

4.3 Cluster Analysis

In this study, the neighbour joining (NJ) analysis (Figure 5) clustered thirty individuals into five major clusters. Some individuals from the same population were clustered together but most of the individuals clustered with other individuals from different populations. A UPGMA (Unweighted Pair Group Method and Arithmetic Average) dendrogram (Figure 6) was subsequently constructed to show the relationships among the populations of *H. macrolepidota*. Three distinct clusters can be identified from the UPGMA tree. The first cluster comprises two populations from Spak River (SP) and Lan River (LA). The second cluster is formed by the populations from Serting River (SR), Menuan River (MR), Pelagus River (PR), Jempol River (JR) and Krang River (KR). The third cluster comprises two populations from Batang Ai (BA) and Loagan Bunut (LB) and the population from Layar River (LR) was basal to the other group.

In general, both NJ tree and UPGMA tree showed a similar topology. UPGMA tree showed that the populations from Peninsular Malaysia (Serting River and Jempol River) were clustered together with the populations from Sarawak; similar with the NJ tree. In addition, individuals from the Menuan River (Kapit) were clustered together with individual from the Pelagus River (Kapit), Jempol River (Negeri Sembilan), Serting River (Negeri Sembilan) or Krang River (Balai Ringin) in the NJ tree. Similarly, the UPGMA tree also showed that the population from Menuan River was closely related with the populations from Serting River; Jempol River; Pelagus River and Krang River.